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Targeted delivery of multivalent phage display vectors into mammalian cells

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Abstract

Novel peptide motives targeting endocytosing receptors were isolated from phage display libraries of random peptides by recovering internalized phage from mammalian cells. The peptide-presenting phage selected by internalization in HEp-2 and ECV304 human cells were taken up 1000- to 100 000-fold more efficiently than their parent libraries, and from 10 to 100 times faster than phage particles displaying integrin-binding peptides. A high degree of selectivity of phage uptake was observed in these cells: phage selected in ECV304 cells were internalized approximately 100-fold more efficiently in ECV304 cells than in HEp-2 cells. Likewise, phage selected in HEp-2 cells were subsequently taken up approximately 40-fold more efficiently by HEp-2 cells than by ECV304 cells. In multiple independent trials using a cyclic peptide library, an identical peptide sequence displayed on phage was internalized by and recovered from ECV304 cells. These findings indicate that the internalization process is highly selective, and is capable of capturing a specific peptide from 2×10^7 peptide variants. Immunofluorescence microscopy showed juxtanuclear localization of internalized phage. These results demonstrate the feasibility of using multivalent phage-display libraries to identify new targeting ligands for the intracellular delivery of macromolecules.

Keywords: Phage display; Phage internalization; Receptor-mediated endocytosis; Targeted delivery

1. Introduction

Receptor-mediated endocytosis is widely exploited in experimental systems as a natural pathway for the targeted delivery of therapeutic agents into cells [1]. In these systems, ligands that bind to target receptors or cell surface antigens are coupled to appropriate drugs, radioisotopes or toxins [2–4]. Ligands can also be attached to delivery vehicles, such as synthetic gene complexes [5], liposomes [6,7], or viruses [8–10]. Upon exposure to cells, these vehicles bind cell receptors and undergo endocytosis. Once inside the cell, the therapeutic agent is transported to a specific intracellular compartment where it exerts its effects. The application of this approach in vivo is limited by several factors – principally the low targeting efficiency of receptor-mediated delivery systems. One way to improve targeting efficiency is to

Abbreviations: FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; HBSS(−), HBSS without Ca^{2+} and Mg^{2+}; IPTG, isopropyl β-D-thiogalactoside; LB, Luria–Bertani medium; PBS, phosphate-buffered saline; TU, ampicillin-resistance transducing unit

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generate novel ligands that would provide a higher specificity and increased rate of internalization. New cell-specific antibodies that exhibit highly selective endocytosis by target cells have been used for this purpose, and have resulted in the development of novel drug delivery systems (for examples see [7,11]). However, the limited repertoire of known cell specific antibodies poses a major limitation on the widespread use of this strategy.

Phage display technology [12,13] provides a new and practically unlimited source of molecular variants, including short peptides [14], antibody fragments [15], and modifications of natural ligands to cell receptors [16,17]. To exploit this technology for the isolation of cell-specific ligands for receptor-mediated intracellular delivery, the phage display vectors should not only be able to bind the cell surface and undergo internalization, but also should be capable of being recovered in an infective form for subsequent rounds of amplification and reselection.

It has been shown that filamentous bacteriophage exposing multiple copies of integrin-binding peptide bind to mammalian cells and undergo internalization as visualized by immunofluorescence microscopy [18]. We have previously demonstrated that phage internalized by mammalian cells enter two distinct subcellular compartments and can be recovered from both these compartments in an infective form [19]. We now report the means for screening phage display libraries and selection of the ligands targeting endocytosed receptors. We describe the application of this approach to isolate phage-displayed peptides that specifically internalized by mammalian cells.

2. Materials and methods

2.1. Materials

Human HEp-2 laryngeal carcinoma and human ECV304 endothelial cell lines were obtained from the American Type Culture Collection. HEp-2 cells were grown in Dulbecco's modified Eagle medium (Life Technologies) supplemented with 10% FBS (Sigma). ECV304 cells were propagated in Medium 199 (Life Technologies) supplemented with 10% of heat-inactivated FBS, and Antibiotic–Antimycotic cocktail (Life Technologies).

The phagemid pC89 and the nonapeptide libraries constructed using this vector in the N-terminal region of the major coat protein pVIII (Fig. 1) were described [20,21].

2.2. Screening of phage libraries for peptides that mediate phage internalization

HEp-2 and ECV304 cells were grown in 150-mm tissue culture dishes (Corning) for 2 days to reach a subconfluent monolayer. Cells were washed with medium supplemented with 10% FBS, and 15 ml of medium was added to each dish. An aliquot of a library containing 3×10^{11} TU was added to the cells and mixed by rocking the plates. Each library was represented by approximately 10^8 independent clones with at least 25% of the clones producing a recombinant protein and thus likely displaying a peptide on the phage surface [20,21]. Therefore, in the aliquot used for screening, each clone was represented by an average of 3000 TU. After the phage were added to the cells, an aliquot of tissue culture medium was saved from each dish to determine the actual phage input. In experiments using chloroquine, cells were preincubated with tissue culture medium supplemented with 10% FBS and 100 μM chloroquine for 30 min at 37°C prior to the addition of phage. Following the incubation of the cells with phage at 37°C for the required period of time (3–24 h), the internalization events were stopped by placing the dishes into ice for 5 min. The following procedures until cell lysis were performed at 4°C. Cells were washed four times with 40 ml of HBSS (Life Technologies), and once with 40 ml HBSS(−). To inactivate the extracellular phage, cells were incubated with 11 ml of subtilisin (3 mg/ml Protease Type XXVII (Sigma) prepared in Buffer I (HBSS(−), 20 mM Tris, 2 mM EDTA, pH 8.0) for 1 h. Cells were detached from the wells by gentle pipetting to achieve a single-cell suspension and then collected into conical-bottom 15-ml centrifuge tubes. To inactivate the subtilisin, cells were pelleted by centrifugation ($200\times g$, 5 min), resuspended in 6 ml of 1 mM PMSF in HBSS(−) containing 2 mM EDTA, and incubated for 15 min. Cells were washed with Buffer I, pelleted again, and the pellets resuspended in 2 ml of lysis buffer (2% sodium deoxycholate (Sigma), 10 mM Tris, 2 mM EDTA, pH 8.0) at room temperature, which was

followed by the addition of 1 ml of TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.5) to each sample. To ensure complete cell lysis and liberation of internalized phage, lysates were vortexed 3 times for 10 s each at maximum speed, pipetted 20 times, and allowed to stay at room temperature for 1 h. An aliquot of each lysate was saved to determine the total number of phage recovered. In order to neutralize EDTA in the lysis buffer and perform infection, 60 μl of 100 mM $CaCl_2$ was added to 5 ml of *E. coli* XLI-Blue bacteria (Stratagene) at late log phase of growth, and then mixed with each cell lysate. The mixtures were incubated for 15 min at 37°C in a water bath without agitation followed by a 30-min incubation with gentle shaking at 80 rpm. Bacteria were collected by centrifugation (4000×*g*, 10 min) and resuspended in 1 ml of LB medium. An aliquot of each bacterial suspension was saved to determine the total number of bacteria infected. Bacteria from each lysate were spread on four 150-mm diameter LB plates containing 100 μg/ml ampicillin and 1% (w/v) glucose and incubated overnight at 37°C. Bacteria colonies were scraped from the plate surface and resuspended in 3 ml of LB per plate.

The phage recovered from the cell lysate were propagated essentially as described [22]. Thirty milliliters of LB containing ampicillin (50 μg/ml) was inoculated with the scraped bacteria to an optical density OD_{600} of 0.05. Cultures were incubated at 37°C with vigorous shaking for about 2 h until $OD_{600} \approx 0.25$ followed by gentle agitation at 80 rpm for an additional 15 min to allow regeneration of pili. Bacteria were then superinfected with M13KO7 helper phage (Pharmacia Biotech) at a multiplicity of infection of approximately 30. IPTG was added at this time (1 mM final concentration). Cultures were incubated for 15 min at 37°C without agitation to allow for infection, aliquoted into test tubes, and incubated at 37°C for 5 h on a roller drum. Following incubation, bacteria were eliminated by centrifugation (4000×*g*, 15 min), and phage particles were precipitated from the supernatant by adding PEG8000/NaCl (5% and 0.27 M final concentrations, respectively) for 1.5 h at 4°C. Phage were pelleted by centrifugation (11 500×*g*, 30 min) and resuspended in 0.015 supernatant volume of TBS buffer. Phage samples were incubated for 30 min at 70°C, and bacterial debris were removed by centrifugation (15 000×*g*, 5 min). Phage titer was determined as described [19]. The preparations were stored at 4°C.

In consecutive rounds of selection, phage input per tissue culture dish was adjusted so that the number of TU recovered from the cell lysate did not exceed 10^5 to 5×10^6.

Phagemid DNA from individual bacterial clones was purified on QIAprep columns (Qiagen). The peptide-encoding DNA inserts were sequenced using M13 (−40) primer (United States Biochemical) with a Prism 377 automated DNA Sequenator and Fluorescent Labeled Dye Deoxy Terminator Chemistry sequencing protocol (PE Biosystems).

2.3. Phage internalization assay

The HEp-2 and ECV304 cells were grown as subconfluent monolayers in 6-well tissue culture plates (Falcon). Cells were incubated with phage for 3 h at 37°C, and the number of internalized intact phage was determined as described [19].

2.4. Immunofluorescence microscopy

HEp-2 and ECV304 cells were grown on 8-well Lab-Tek chamber glass slides (Nunc). Following incubation of the cells with phage for 5 h at 37°C, the internalized phage particles were visualized by immunostaining as described [19].

3. Results

3.1. Selection of phage that undergo internalization by tissue culture cells

To identify novel peptide ligands targeting endocytosing receptors, we used libraries of random peptides displayed on pC89 phagemid particles (Fig. 1). Libraries in this particular vector were chosen because of a relatively small length of these phage (ca. 0.5 μm), and a multivalent mode of peptide presentation, which we have previously shown to be principally important for effective phage uptake [19]. In order to screen the libraries, the procedure that had been developed for the quantitation of internalized intact phage [19] was scaled up. Our initial

```
AEGDDPAK...          (wt)

AEGEFCXXXXXXXXXXCGDPAK...    pVIII-9aa.Cys
      └──s-s──┘

AEGEFXXXXXXXXXXDPAK...       pVIII-9aa
```

Fig. 1. Amino acid sequence of the N-terminal portion of the wild-type major coat protein (pVIII) and the recombinant pVIII containing random oligopeptide inserts. The randomized residues are marked with (X). In the pVIII-9aa.Cys library [21], the random peptide sequences are flanked by two cysteines to favor the presentation of the peptides in a conformationally constrained cyclic form. The putative disulfide bond is indicated. In the pVIII-9aa library [20], the random peptides are expressed in a linear form. Both libraries are represented by approximately 2×10^7 independent clones encoding random peptide sequences [20,21]. The amino acid sequence of inserted oligopeptides is shown in bold.

selection experiments showed that the ratio of the number of internalized phage to the number of phage added increased rapidly and reached a maximum value after three or four rounds of selection (Fig. 2A). Approximately 25% of clones in the original library contained productive peptide inserts that could be easily identified on β-galactosidase indicator plates [21]. After two rounds of selection, the portion of peptide-containing clones rapidly increased to 90–98% demonstrating effective selection of insert-containing phage. Similar results were obtained in selection experiments performed under different conditions using the library of random cyclic peptides (Fig. 2A–C). After three rounds of selection, isolated phage populations were internalized from 1000- to 100 000-fold more efficiently than the parent library. The linear peptide library also provided rapid isolation of internalized phage after three rounds of selection (Fig. 2D). Since the conformationally constrained cyclic peptides generally demonstrate higher affinity for target receptors than unconstrained linear peptides [13,23], we performed further experiments with pVIII-9aa.Cys library of cyclic peptides.

We next investigated whether specific selection conditions would favor isolation of phage that undergo different intracellular routing. The use of chloroquine, a lysosomotropic agent, would be expected to favor survival and recovery of phage transported to lysosomes. On the other hand, prolonged incubation with phage would be expected to facilitate the accumulation of phage that evade lysosomes. We performed selection in the presence (Fig. 2A) or absence (Fig. 2B,C) of 100 μM chloroquine, and with 3 h (Fig. 2B) or 24 h (Fig. 2C) incubation periods. To trace the intracellular fate of isolated phage populations, we measured the effect of chloroquine on the titer of recovered phage (Fig. 3). Phage populations isolated after three rounds of uptake by HEp-2 cells in either the presence (Hch3-phage) or absence (H3-phage) of chloroquine were then incubated with

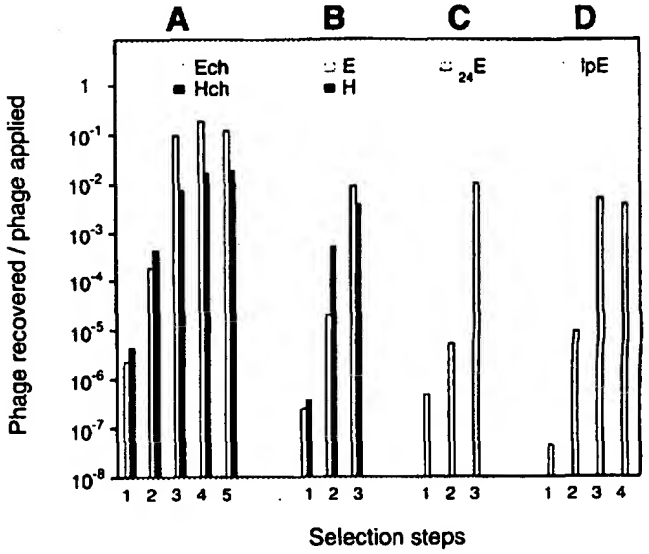


Fig. 2. Enrichment in the yield of internalized phage in consecutive selection steps. ECV304 or HEp-2 cells were incubated with a random peptide library for a specified period of time at 37°C. The extracellularly bound phage were inactivated by subtilisin treatment. Internalized phage were recovered by lysis of the cells, and were used for propagation in bacteria and reselection as described in Section 2. The numbers of consecutive selection steps are plotted on the *x*-axis. Peptide-bearing phage were selected in four independent experiments from either the cyclic peptide library, pVIII-9aa.Cys (A–C), or the linear peptide library, pVIII-9aa (D). The bars represent a fraction of the applied phage population recovered from ECV304 (open bars, □) or HEp-2 (filled bars, ■) cells. (A) Cells were incubated with the cyclic peptide library for 5 h in the presence of 100 μM chloroquine. Phage populations recovered from ECV304 and HEp-2 cells are designated as *Ech* and *Hch*, respectively. (B) Cells were incubated with the cyclic peptide library for 3 h in the absence of chloroquine. Phage populations recovered from ECV304 and HEp-2 cells are designated as *E* and *H*, respectively. (C) ECV304 cells were incubated with the cyclic peptide library for 24 h in the absence of chloroquine. Populations of phage recovered in cell lysates are designated as *24E*. (D) ECV304 cells were incubated with the linear peptide library for 3 h in the absence of chloroquine. Phage populations recovered in cell lysates are designated as *lpE*.

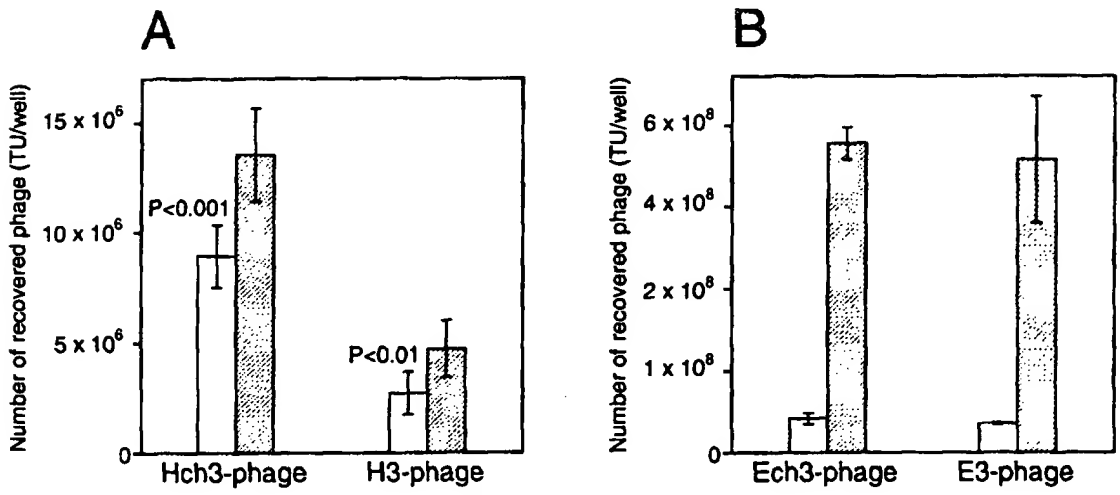
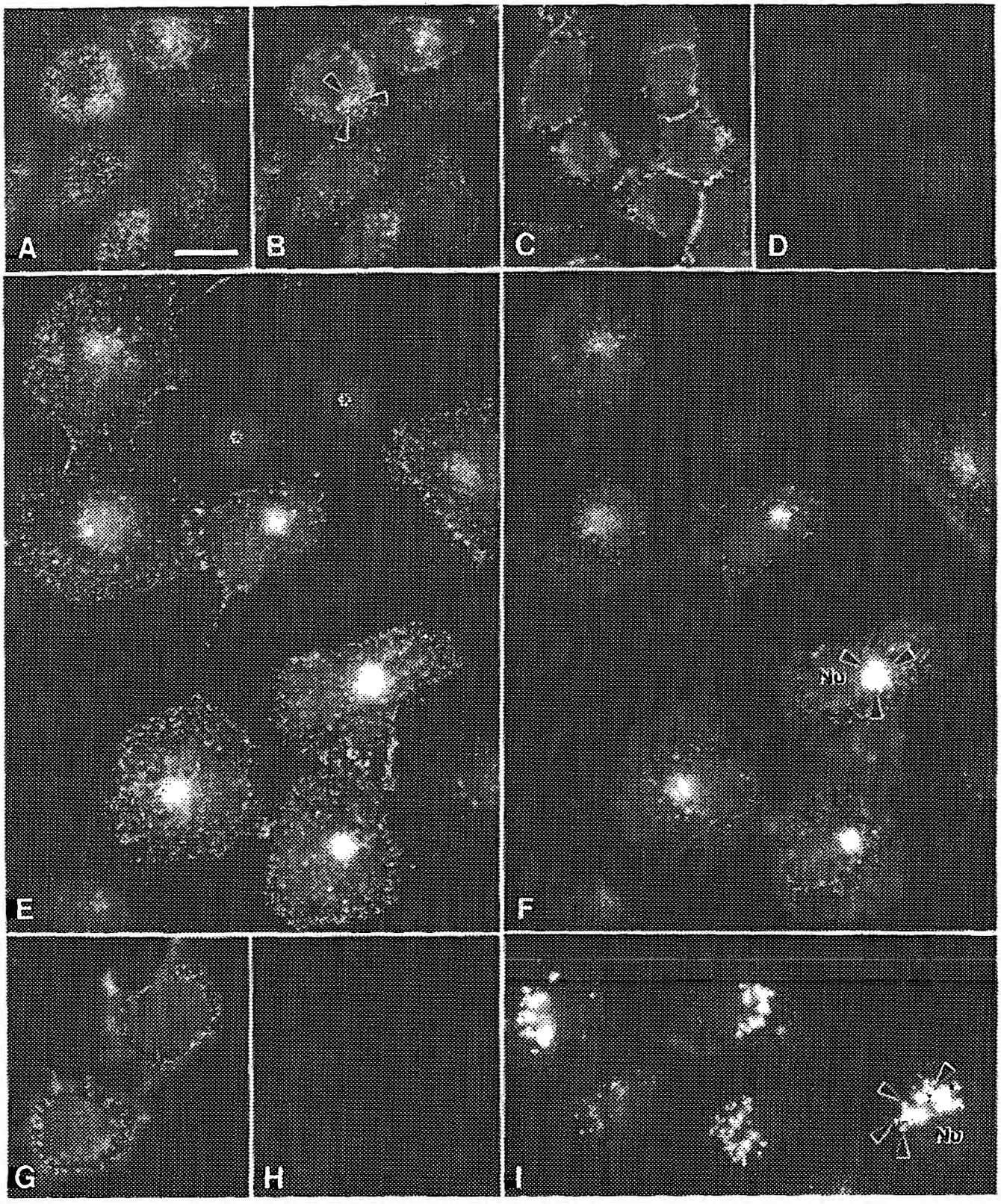


Fig. 3. Internalization of selected phage populations by HEp-2 and ECV304 cells, and the effect of chloroquine on recovery of internalized phage. Phage populations are designated as in the legend to Fig. 2. The following number corresponds to the selection step (e.g. the phage population designated as Hch3-phage was obtained after the third round of selection of pVIII-9aa.Cys library on HEp-2 cells as shown in Fig. 2A). HEp-2 cells (A) and ECV304 cells (B) were grown in 6-well plates to a subconfluent monolayer, and then incubated with the indicated phage population (2×10^{10} TU/well) for 3 h at 37°C in the absence (open bars) or presence (hatched bars) of 100 μM chloroquine. The number of internalized intact phage was assayed as described [19]. Results are shown as the mean ± S.D. of phage yield from 9 wells, and are representative of two experiments. Statistical analysis was performed using unpaired Student's *t* test.

HEp-2 cells to determine whether chloroquine would cause different effects on titer of recovered phage. As shown in Fig. 3A, chloroquine increased the phage titer 1.5–1.6 times in both phage populations. Similarly, phage populations selected after three rounds of uptake by ECV304 cells in either the presence (Ech3-phage) or absence (E3-phage) of chloroquine were then incubated with ECV304 cells (Fig. 3B). Chloroquine increased the phage yield approximately ten-fold for both *Ech3* and *E3* phage populations. These data suggest that phage populations isolated in the presence or absence of chloroquine undergo similar intracellular fates. In contrast, the two cell lines differed in the effect of chloroquine on the survival of internalized phage: phage yield increased from 1.5 to 1.6 times in HEp-2 cells (Fig. 3A), and up to 10-fold in ECV304 cells (Fig. 3B). These results suggest that internalized phage undergo different routing in each cell line, directed primarily to lysosomes in ECV304 cells, and directed primarily to a non-lysosomal compartment(s) in HEp-2 cells. While chloroquine can exert non-lysosomal effects in mammalian cells (e.g. [24]), it is well documented that lysosomes are a major site of chloroquine accumulation [25]. Within lysosomes, chloroquine increases internal pH and thereby inhibits lysosomal hydrolases and prevents degradation of macromolecules [25]. Our fluorescence microscopy study revealed that chloroquine prevented processing of internalized phage into fine scattered particles, but rather promoted accumulation of phage into large aggregates (Fig. 4I, compare to Fig. 4F). It is likely that the major effect of chloroquine on the viability of internalized phage could be attributed to the inhibition of phage degradation in lysosomes, although other possibilities cannot be formally excluded.

3.2. *Characterization of the isolated peptides that mediate phage internalization*

DNA from individual clones of isolated phage was sequenced to infer amino acid sequence of displayed peptides (Table 1). In the *Hch3* phage population that was selected after three rounds of internalization by HEp-2 cells in the presence of chloroquine, a phage isolate (1) was obtained multiple times suggesting that this particular peptide was more readily taken up by the cells. After two additional rounds of selection, the isolates from the *Hch5* phage population exhibited a common motif consisting of glycine


A
B
C
D
E
F
Nu
G
H
I
Nu

residues occupying the fifth, seventh, and ninth positions, and a hydrophobic residue in the eighth position (Table 1). In the independent selection experiment performed with HEp-2 cells in the absence of chloroquine, different peptides were identified within the *H3* phage population. However, they exhibited a motif resembling that from the *Hch5* isolates. Thus, all isolates from the *H3* population had glycine in the seventh and ninth positions, and a hydrophobic residue in the eighth position, which is similar to the motif found in the *Hch5* isolates. The same peptide sequence was isolated in two independent selection experiments (Table 1, inserts 6 and 8). Taken together, these results suggest that, regardless of the selection conditions, phage-displayed peptides target the same or very similar receptors on the surface of HEp-2 cells.

In three independent selection experiments performed under different conditions with ECV304 cells, identical peptides were identified in phage populations *Ech3*, *E3*, and $_{24}$*E3* after three rounds of internalization (Table 1). Two peptides isolated within each phage population showed high amino acid similarity. In both peptides, positions one, two, three, seven, and nine were occupied by the identical amino acid residues, and positions four and five were occupied by conservative hydrophobic and positively charged residues, respectively. The data demonstrate a remarkable selectivity of the internalization process in ECV304 cells resulting in the isolation of two particular peptides from approximately 2×10^7 variants. These results also suggest that peptides selected under different conditions target the same receptors

Table 1
Amino acid sequence of the isolated peptides that mediate phage internalization

Phage population	Insert	Amino acid sequence of the insert
		123456789
Hch3	1 (3)	[AEGEFC]-RLTGGKGVG-[CGDPAK...]
	2	TSGFSYGAW
	3	IYPSYGTTL
	4	TLYGSRGVG
Hch5	5 (2)	RLTGGKGVG
	6	QISHGTGIG
H3	7 (2)	ARHGSSGAG
	8	QISHGTGIG
	9	TREPFGVG
Ech3	10 (3)	SPLWRNSHL
	11	SPLFKHSVL
E3	12 (3)	SPLWRNSHL
	13	SPLFKHSVL
$_{24}$E3	14 (3)	SPLWRNSHL
	15	SPLFKHSVL

Phage populations are designated as described in legends to Figs. 2 and 3. *Hch3* and *Hch5* correspond to phage populations isolated after three and five rounds of selection, respectively, in the same selection experiment (Fig. 2A). All other phage populations were isolated in independent selection experiments (Fig. 2). Phagemid DNA from 25 individual bacterial clones was purified, sequenced, and the amino acid sequence of the peptide insert was deduced. If greater than one, the number of occurrences of a particular insert is indicated in parentheses. Conventional single letter amino acid code is used. Only the variable inserts of nine amino acid residues located between two cysteines are shown. Position of these residues is numbered at the top. For insert 1, the invariable flanking sequences presented in all pVIII-fusion isolates are shown in the brackets. In insert 9, an in-frame deletion of one residue was detected. Within each phage population, the same residues found in peptide inserts in the same position are underlined.

←

Fig. 4. Detection of internalized phage particles by immunofluorescence microscopy. The cells were grown on chamber slides and incubated with Hch5-phage or Ech3-phage (2×10^9 TU/well) for 5 h at 37°C. The slides were then processed for immunodetection of phage particles as described in Section 2. Hch5-phage were incubated with HEp-2 cells (A–C) and ECV304 cells (D), followed by immunostaining. Localization of phage particles bound to the surface of HEp-2 cells was demonstrated in the optical plane through the very apical region of the cells (A), whereas the internalized phage were revealed in the optical planes through the middle of the same cells (B), as indicated by arrowheads. In C, permeabilization of the cells with saponin was omitted in order to identify extracellular bound phage only, and the optical plane through the middle of cells is shown. The background fluorescence reveals the cell nuclei in D, and also in E–I. Ech3-phage were incubated with ECV304 cells (E–G) and HEp-2 cells (H), followed by immunostaining. E and F Show the same cells in two optical planes: at the level of flattened periphery of the cells (E) and through the cell nuclei (F). Punctuate immunofluorescence of phage particles bound to the surface of cells is shown in E. Two cells whose nuclei are marked with asterisks (E) demonstrate no binding or uptake of phage. Internalized phage clustered on one side of the cell nuclei are revealed in the optical plane through the cell nuclei, as indicated by arrowheads (F). In G, permeabilization of ECV304 cells was omitted, and the optical plane through the cell nuclei is shown. (I) ECV304 cells were incubated with Ech3-phage in the presence of 100 μM chloroquine followed by immunostaining. Arrowheads in I indicate large accumulations of internalized phage; Nu, cell nucleus. Scale bar: 20 μm.

Table 2
Selectivity of Ech3-phage and Hch5-phage internalization by ECV304 and HEp-2 cells

Phage population	Number of phage recovered from ECV304 cells (TU per well)	Number of phage recovered from HEp-2 cells (TU per well)	Ratio of the means: phage recovered from ECV304 cells/phage recovered from HEp-2 cells
Ech3-phage	$(16.50 \pm 2.7) \times 10^6$	$(0.19 \pm 0.067) \times 10^6$	97:1
Hch5-phage	$(0.061 \pm 0.014) \times 10^6$	$(2.96 \pm 0.12) \times 10^6$	1:37

ECV304 and HEp-2 cells grown in 6-well plates to a subconfluent monolayer were incubated with the indicated phage population (2×10^{10} TU/well) for 3 h at 37°C. The number of internalized intact phage was assayed as described [19]. The data are the means ± S.D. from three wells and are representative of three experiments.

on the surface of ECV304 cells, which probably mediate the highest rate of phage uptake.

3.3. Selectivity of peptide-mediated phage uptake

Ech3-phage were internalized by ECV304 cells about 100-fold more efficiently than HEp-2 cells (Table 2). Likewise, Hch5-phage were taken up by HEp-2 cells approximately 40 times more efficiently than ECV304 cells. These data show that although no attempts were taken to restrict the internalization of isolated phage to a particular cell type, the selectivity of uptake was quite high.

3.4. Immunodetection of phage particles

Immunodetection allowed visualization of individual phage particles. In preliminary experiments, phagemid pC89 and helper phage M13KO7 particles were immobilized on polylysine-coated slides and immunostained. The phagemid particles of approximately 0.5 μm in length were revealed as bright oblong dots, whereas M13KO7 phage of approximately 1.2 μm were resolved as elongated rods (data not shown).

Hch5-phage bound strongly to the surface of HEp-2 cells (Fig. 4A). Internalized particles were detected within the cells in the optical planes through the middle of the cells (Fig. 4B). In the cells processed for immunostaining without prior permeabilization, only punctuate staining of extracellularly bound phage on adjacent plasma membranes was detected (Fig. 4C). No appreciable binding or internalization of Hch5-phage was demonstrated by ECV304 cells (Fig. 4D).

Ech3-phage were bound and internalized by ECV304 cells (Fig. 4E,F), the internalized phage being predominantly localized to one side of the cell nucleus (Fig. 4F). The immunolocalization of Ech3-phage in individual cells was strikingly heterogeneous, showing strong binding and internalization of phage in the majority of the cells and lack of binding and uptake in other cells (Fig. 4E,F). Only a few cells demonstrated an intermediate level of binding and internalization. In non-permeabilized ECV304 cells, extracellularly bound phage appeared as elongated 'aggregates' (Fig. 4G). HEp-2 cells demonstrated no significant binding or internalization of Ech3-phage (Fig. 4H). Chloroquine dramatically affected the distribution of Ech3-phage in ECV304 cells showing large elongated or circular accumulations of intracellular phage (Fig. 4I) when compared with the scattered fine granular pattern of the phage particles internalized in the absence of chloroquine (Fig. 4F). The immunolocalization results show that the peptides isolated from the phage display library mediate selective intracellular delivery of phage particles in different cell types.

4. Discussion

Previous studies have demonstrated the isolation of cell-binding peptides by in vitro and in vivo application of phage peptide libraries [26–30]. We report here a new selection strategy for the isolation of cell-entering peptides which mediate internalization of phage vectors in a cell-type specific manner. A potential use for such selection strategy is in the identification of 'targeting' ligands that could mediate intracellular delivery of macromolecules.

We have employed two innovations which seem to be important for the effective selection of internalizable phage-displayed ligands: use of multivalent

phage display vectors and inactivation of extracellular-bound phage with subtilisin. Presentation of receptor-binding peptides on the phage surface in a multivalent mode may be a prerequisite for effective phage uptake [19], because it is likely that pC89 phage of this size (0.5 μm length) are internalized via a mechanism resembling phagocytosis and involving multipoint 'zipper' contacts of the phage particle with cell surface receptors [31]. Thus, although cell-selective binding peptides have been isolated from phage display libraries in several studies [26–29], no significant internalization of the bound phage has been reported [27,29]. The lack of phage uptake in these studies may be due to the expression of the peptides as fusions with pIII at low copy numbers per phage particle that are insufficient for multipoint interactions with cellular receptors.

The second innovation, the use of the protease subtilisin to inactivate extracellular phage, addresses the issue of how solely internalized phage could be recovered from the cells for amplification and subsequent rounds of reselection. We have shown that the incubation of bound phage with subtilisin reduced a portion of extracellular phage recovered together with internalized phage in cell lysate to less than 0.1% [19]. Thus, treatment with subtilisin effectively inactivate extracellularly bound phage and allows maintenance of viable internalized phage.

The selection scheme used in our study favored isolation of peptides that facilitated the highest rate of phage uptake. It is likely that these peptides bind cell surface receptors that are expressed in abundance and which undergo rapid internalization. Other peptide variants targeting less abundant and/or slower internalized receptors were depleted during selection. For example, the RGD peptide mediates phage internalization at ten to a hundred times lower rate [19] than phage populations described in this study. This may explain why we were unable to identify RGD-containing peptides in the pools of selected phage. In general, the most efficiently internalized peptide-on-phage are not necessarily the most selective for a particular cell type. Therefore, in order to isolate the most selective ligands, protocols in which phage that bind other cell types are subtracted from phage internalized by the cell type of interest should be employed.

The highly selective manner in which peptide-on-phage are internalized by cells may also offer a means to identify subpopulations of cells that differ in cell surface determinants. This seems to be the case for the phage populations *Ech3*, *E3*, and $_{24}E3$ (see Table 1) which showed heterogeneous binding and internalization by individual ECV304 cells (see Fig. 4E,F). A similar pattern of heterogeneous immunolocalization has been reported for phage selected for binding to fibroblasts [27]. Although some cells demonstrated intermediate level of immunostaining, most of the cells bound and internalized phage in an all or none fashion. This heterogeneity appears not to be related to the ability of the cells to internalize phage since phage particles displaying integrin-binding peptides were taken up uniformly by the individual cells (data not shown). Therefore, it is likely that ECV304 are heterogeneous for the receptor that recognizes the isolated peptide-on-phage. The expression and/or availability of this receptor for binding phage seems not to correlate with the phase of the cell cycle, since incubation of the cells with phage for either 3 or 21 h resulted in approximately the same ratio of stained and non-stained cells (data not shown). The heterogeneity of ECV304 cells revealed by immunostaining of phage suggests a high resolution power of the technique in distinguishing subpopulations of endothelial cells in a tissue culture model.

In conclusion, our results demonstrate feasibility of exploiting multivalent phage display libraries for the selection of novel peptide ligands that target endocytosing receptors and enter the cells. Optimization of the existing vectors would facilitate effective isolation of such ligands and simplify their transplantation into the appropriate delivery vehicles. This may allow the generation of advanced targeting systems for intracellular delivery of macromolecules.

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